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# METABOLIC ACTIVATION OF DIETHYLSTILBESTROL: INDIRECT EVIDENCE FOR THE FORMATION OF A STILBENE OXIDE INTERMEDIATE IN HAMSTER AND RAT

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The synthetic estrogen diethylstilbestrol (DES) has been associated with the occurrence of vaginal and cervical tumors in young women whose mothers had received DES medication during pregnancy (1,2). Among the data available for the carcinogenic activity of DES in animals (3), the high incidence of renal tumors in male hamsters is evident (4,5). Furthermore, binding of DES to tissue macromolecules has been related to hepatic centrolobular necrosis. It has been shown by in vitro experiments that this binding requires a reactive intermediate generated from DES by a cytochrome P-450 dependent oxidation (6,7).

Studies carried out in our laboratory about the biotransformation of carcinogenic trans-4-dimethylaminostilbene and its inactive cis-isomer revealed that epoxidation of the stilbene double bond represents a major pathway for the in vivo metabolism of stilbene amine derivatives (8). Since an epoxide of DES as a reactive intermediate metabolite could possibly account for the toxic properties, we are presently investigating the in vivo metabolism of DES in laboratory animals. In this communication we wish to report the identification of six new metabolites from urine and bile of hamster and rat, some of which can be taken as evidence for the metabolic epoxidation of the stilbene double bond in DES.

The study was carried out using a mixture of [G-$^{3}$H]-DES obtained from the Radiochemical Centre Amersham and [monoethyl-1.2-$^{2}H_5$]-DES prepared from perdeutero-ethanol in this laboratory by a modification of the methods of Dodds and Kuwada (9,10). Male Syrian golden hamsters (150-200 g body wt.) and female Wistar rats (200-250 g body wt.) were injected i.p. with 50 mg/kg [$^{3}$H,$^{2}$H]-DES dissolved in 0.5 ml of propane-1,2-diol. Urine was collected in 24-hour periods for 5 days and stored at -20°C. Bile was obtained from unanesthesized cannulated

Abbreviations: DES, diethylstilbestrol; GLC, gas liquid chromatography; TLC, thin layer chromatography; TMS, trimethylsilyl.

rats (10 mg/kg) and from the gall bladder of hamsters (50 mg/kg), respectively. The extraction of radioactive metabolites from urine and bile, their separation into unconjugated and conjugated compounds by chromatography on alumina and the enzymatic hydrolysis of glucuronides were performed as previously described for aminostilbenes (11). Radio-GLC (12,13) was used to trace the radioactive metabolites prior to their identification by GLC-mass spectrometry (for details see Table 1).

Urinary excretion of radioactivity within 5 days after i.p.administration of 10 mg of [$^{3}$H,$^{2}$H]-DES was 20-30% of the dose both in the hamster and in the rat, the radioactivity being predominantly (60-70%) associated with glucuronides. The pattern of metabolites found after hydrolysis in the urine from hamsters and rats differed considerably. DES represents the major urinary glucuronide of the hamster, but could not be detected in significant amounts in rat urine. The main glucuronide excreted by the rat is DES carrying an additional hydroxy-group (metabolite B, see Fig.1) which according to the mass spectrum can not be located at the aliphatic moiety.

A major metabolite (metabolite E) in both rat and hamster urine was found to contain an additional double bond. The identity of metabolite E with dienestrol (see Fig.1) had to be inferred from the mass spectrum which, compared to

Table 1. Urinary and biliary metabolites of DES excreted as glucuronide conjugates by the hamster and by the rat.

| | Metabolite | GLC[a] retention time | $M^+$ in mass spectrum[b] | Content[c] in urine: hamster | rat |
|---|---|---|---|---|---|
| A | DES | 12.4/14.6 | 412/417 | 4.7 | n.d.[d] |
| B | hydroxy-DES | 16.7/18.0 | 500/505 | n.d. | 6.0 |
| C | methoxy-DES | 15.2/17.9 | 442/447 | n.d. | n.d. |
| D | dimethoxy-DES | 18.8/20.9 | 472/477 | n.d. | n.d. |
| E | dienestrol | 18.8 | 410/414 | 0.8 | 2.7 |
| F | hydroxy-dienestrol | 22.4 | 498/502 | 3.2 | 0.5 |
| G | methoxy-dienestrol | 21.4 | 440/444 | n.d. | 1.3 |

a) of metabolites after trimethylsilylation with bis(O.N)-trimethylsilyl-acetamide; glass column 6 ft.x 1/4", 2 mm i.d., 3% OV-225 on GasChrom Q 100/120 mesh, He 30 ml/min., temperature program 160-260°C with 4°C/min. The two peaks found with stilbenes correspond to the cis- and trans-isomer.

b) the molecular ions $M^+$ refer to the TMS-derivatives; the twin signals are due to the deuterated and non-deuterated DES employed as a 1:1 mixture.

c) % of dose excreted within 5 days; the values are semiquantitative since only a few animals were used.

d) not detected.

DES, exhibited a molecular ion shifted to lower mass by two mass units for the non-deuterated molecules and by three mass units for the deuterated species. The complete loss of only one $^{2}H$ precluded an asymmetrical position of the two double bonds. The structure was further substantiated by identical GLC retention time and mass spectrum of metabolite E with one of the isomers of dienestrol obtained by the synthesis of a sample containing all three cis/trans isomers (9). Metabolite E is not the dienestrol isomer employed as synthetic estrogen. In addition, radiochemical pure metabolite E isolated from rat urine by TLC was catalytically hydrogenated. The product obtained was identical with hydrogenated DES with respect to GLC retention time and mass spectrum.

As shown in Table 1, two other metabolites (metabolite F and G) derived from dienestrol by carrying an additional hydroxy- or methoxy-group were identified in urine.

Biliary excretion of radioactivity in the rat within 18 hours accounted for 60-70% of the dose. In hamsters, the gall bladder contained after 3 hours 1-2% of the dose. In both species, biliary radioactivity was almost exclusively (70-80%) associated with the glucuronide fraction. After hydrolysis, about half of the radioactivity was attributed to DES by radio-GLC. Another major metabolite (metabolite C, 20-30%) is derived from DES by an increase of 30 mass units, most

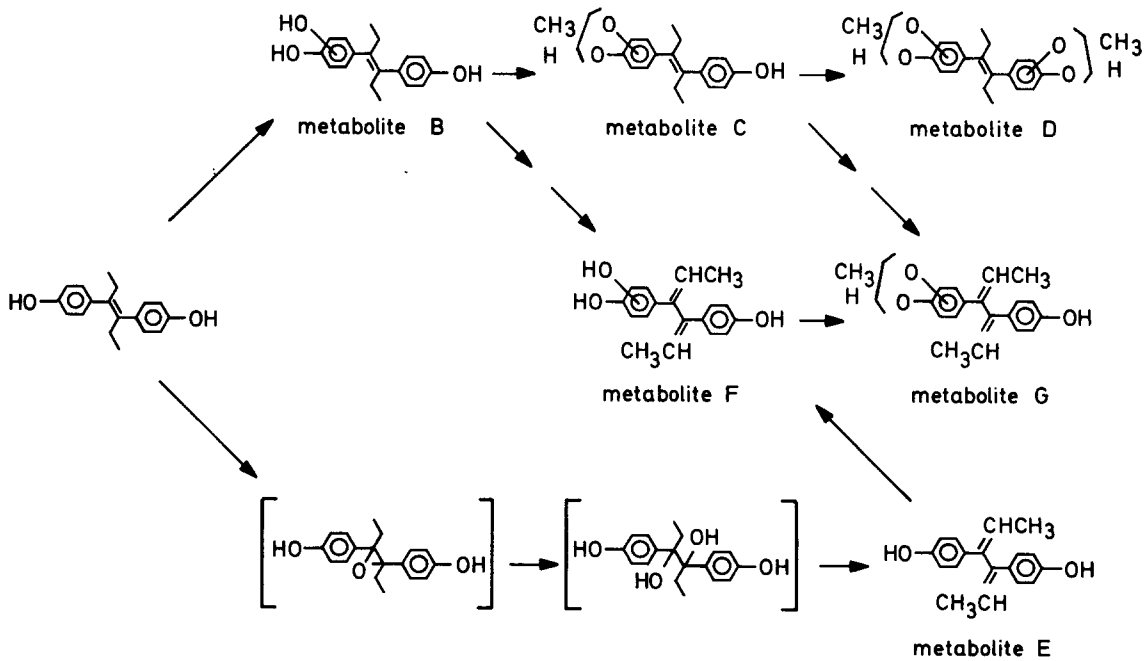


Fig.1. Metabolic pathways of DES in hamster and rat.

likely due to a methoxy-group. A respective dimethoxy-DES (metabolite D, 20%) was present only in rat bile. Catalytic hydrogenation of this metabolite and mass spectrometry of the resulting bibenzyl-derivative showed that the methoxy-groups are located in different aromatic rings.

Besides these methoxy-compounds two other biliary metabolites were identified as hydroxy-DES (metabolite B, 5%) and dienestrol (metabolite E, 10-20%).

Despite the wide application of DES both as synthetic estrogen in human therapy and in food-producing animals (14), the only in vivo metabolite known today is the glucuronide of DES (15,16). The presented data show, however, that DES in addition is extensively oxidized in hamster and rat. One pathway obviously involves hydroxylation of the aromatic ring and methylation of one of the phenolic hydroxy-groups (see Fig.1). The resulting methoxy-DES is exclusively excreted through the bile. Although the exact structures of hydroxy- and methoxy-DES have not yet been established, it may be assumed that the functional groups are in ortho position by analogy to known metabolites of trans-stilbene (17,18) and biphenyl (19). Furthermore, in an in vitro study with rat liver microsomes, the formation of 3-hydroxy-DES and 3-methoxy-DES has been reported (20).

The second pathway involved in DES metabolism affects the stilbene double bond leading to dienestrol and other metabolites with a hexadiene structure. The formation of these metabolites can best be explained by assuming a metabolic epoxidation of the stilbene double bond followed by hydrolysis and loss of water from the resulting diol (see Fig.1). The intermediate epoxide and diol however, have not been detected among the main metabolites. Efforts to analyse the minor metabolites are in progress as are experiments to rule out an alternative route leading to dienestrol by hydroxylation of DES in the allylic position with subsequent loss of water via an allylic rearrangement.

The presented identification of metabolites with hexadiene structure strongly supports the view that epoxidation of the stilbene double bond represents a major pathway of DES metabolism. The epoxide intermediate would have to be considered a reactive metabolite and may well be related to the carcinogenic potential of DES. In addition, the possible toxicological hazard exerted by the formation of catechol derivatives has to be taken into account.

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